Junctional Epidermolysis Bullosis: Defects in Expression of Epiligrin/Nicein/Kalinin and Integrin β4 That Inhibit Hemidesmosome Formation

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Junctional epidermolysis bullosis (JEB) is a heterogeneous inherited blistering disorder of human epithelial basement membranes (BMs). Characteristically, the epidermis detaches from the BM between the basal cells and the lamina lucida due to reduced numbers of hemidesmosomes (HDs). Attempts to identify a candidate gene for JEB led to the characterization of nicein, a protein complex in normal BMs that is absent from BMs of patients with JEB gravis. In independent research, two related BM glycoproteins, epiligrin and kalinin, were identified as functional adhesion components of HDs. Epiligrin was characterized as a BM ligand for basal cell adhesion via integrins α3β1 in focal adhesions and α6β4 in HDs. Kalinin was characterized as an adhesive ligand and a component of anchoring filaments. Recent antibody and sequence studies on epiligrin/nicein/kalinin have identified limited homologies with laminin. Ongoing studies in multiple laboratories seek to identify mutations in one or more of the three subunits of epiligrin that are causal in JEB gravis. Consistent with the genetic heterogeneity of JEB, we have identified a patient with a variant form of JEB that is associated with pyloric atresia. This patient has negligible HDs, normal epiligrin, but reduced expression of integrin β4. A defect in the β4 expression may define a subset of JEB cases that also present with pyloric atresia. These results testify to the dual requirements for epiligrin in the BM and integrin β4 in the plasma membrane in regulating function of HDs in epithelium. ***Key words: epidermis/cell adhesion/integrins/vesiculobullous skin diseases. J Invest Dermatol 103:31S–38S, 1994***

JUNCTIONAL EPIDERMOLYSIS BULLOSIS: DEFECTS IN HEMIDESMOSOMES OF BASAL EPIDERMAL CELLS

Junctional epidermolysis bullosis (JEB) is a blistering disorder of human epithelium and is inherited as an autosomal recessive mutation(s) [1,2]. The clinical findings of blister formation with mild mechanical trauma to the epidermis occurs as a result of separation of viable basal cells from the lamina lucida of the basement membrane (BM). At the ultrastructural level, the patients exhibit reduced numbers, or disorganization of hemidesmosomes (HDs) in the basal plasma membrane of the basal cells. Currently, there are seven variant forms of JEB recognized by the National Epidermolysis Bullosa Registry [1]. These variants range from the milder localized JEB inversa to the severe generalized JEB gravis/Herlitz form (formerly lethal/letalis). In this most severe form, HDs are usually severely reduced or absent in both lesional and unaffected areas of the epidermis. Together these results suggest that i) a genetic defect in an adhesion component(s) of HDs may be causal in JEB, and ii) that JEB is genetically heterogeneous with possible defects in more than one adhesion component.

We identified a new BM glycoprotein, epiligrin, as the major component of the extracellular matrix (ECM) synthesized by cultured human foreskin keratinocytes (HFKs) [3]. Epiligrin is an effective ligand for adhesion of cultured keratinocytes and is a physiologically important mediator of basal cell adhesion to the BM. During the course of our studies on cell adhesion to epiligrin, it became apparent that epiligrin was related or identical to at least two other BM proteins [4], nicein [5,6] and kalinin [7]. Each of these three protein complexes had been identified independently; epiligrin as an adhesive ligand for integrin adhesion receptors α3β1 and α6β4 [3], kalinin as a component of the anchoring filaments associated with HDs [7], and nicein/BM600/GB3 antigen, as a protein antigen that was absent from the BM of patients with the gravis form of JEB [5,6].

Knowledge of the structure/function of normal adhesion components has led to an understanding of the molecular basis of inherited and acquired blistering disorders of the epidermis. In the following two sections we will outline our current understanding of the molecular basis of normal cell-BM and cell-cell adhesion in the basal cell layer of the epidermis (see **Fig 1** for a diagrammatic outline). Then we will evaluate candidate genes for JEB in relation to the components and mechanisms that mediate normal keratinocyte adhesion to the BM.

CELL ADHESION IN BASAL CELLS OF THE EPIDERMIS

Integrin and Cadherin Receptors in Anchoring Junctions

Cell-cell and cell-substrate adhesion in the epidermis is mediated by two types of anchoring junctions termed i) adherens junctions including focal adhesions (FAs) [8] and ii) HDs [9] and desmosomes [10]. Each anchoring junction consists of an extracellular ligand or co-receptor, a transmembrane adhesion receptor(s), and an associated cytoskeletal complex. Desmosomes and HDs are stable anchoring junctions and mediate cell-cell and cell-BM adhesions, re-

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Abbreviations: FA, focal adhesion; HFK, human foreskin keratinocyte; PA, pyloric atresia.

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spectively. They are associated with intermediate filaments [11]. In contrast, adherens junctions are linked to actin containing-filaments [8] and are more dynamic and labile than desmosomes or HDs. Specialized forms of adherens junctions can mediate either cell-cell or cell-substrate adhesion. The integrin class of adhesion receptors are functional in FAs and HDs that mediate basal keratinocyte adhesion to the BM. Cadherin adhesion receptors are present in cell-cell adherens junctions and desmosomes that mediate cell-cell adhesion in the epidermis [10].

Cell-Substrate Adhesion: Integrins in Hemidesmosomes and Focal Adhesions The integrins are the primary adhesion receptors present in both FAs and HDs. Furthermore, FAs and HDs are the primary mediators of basal keratinocyte adhesion to the substratum. Integrins are heterodimeric adhesion receptors that contain dissimilar α and β subunits (for reviews see [12,13]). There are currently 13 different α subunits that associate with eight different β subunits. Normal human skin and cultured HFKs express high levels of integrins $\alpha6\beta4$, $\alpha3\beta1$, and $\alpha2\beta1$ [14]. In addition, keratinocytes in culture express detectable quantities of integrins $\alpha1\beta1$, $\alpha5\beta1$, $\alpha6\beta1$, $\alpha v\beta5$, and $\alpha v\beta6$, in part due to the activation-dependent expression of these receptors in culture and in wounds [15,16]. Based on the described receptor-ligand interactions for many cell types the possible ligand-specificities for the major epidermal integrin receptors are (for review see [17]) $\alpha2\beta1$, collagen [18] and laminin; $\alpha3\beta1$, epiligrin [3], fibronectin, collagen, entactin and laminin; $\alpha6\beta4$, epiligrin [3], and laminin. However, studies with HFKs indicate that the preferred ligand-specificities are $\alpha2\beta1$, collagen; $\alpha3\beta1$, epiligrin; and $\alpha6\beta4$, epiligrin or an epiligrin associated component [3].

HDs are the primary cell-substrate adhesion structures identifiable in epidermis at the ultrastructural level, but are more difficult to detect in cell culture [14]. In skin, HDs are identified by electron microscopy as dense plaques localized to the cytoplasmic side of the basal membrane of keratinocytes attached to the BM. Immature forms of HDs have been identified in culture [11] and referred to as stable anchoring contacts (SACs) [14].

In the basal plasma membrane, HDs contain integrin $\alpha6\beta4$ as a transmembrane adhesion receptor [14,17–19]. In the cytoplasm, HDs contain a 230-kDa form of the bullous pemphigoid antigen (BPAG1) as a component of the electron dense plaque [11]. BPAG1 is structurally and functionally related to desmoplakin I [11], a component of the cytoplasmic plaque of desmosomes. BPAG2 is a unique transmembrane 180-kDa antigen that also localizes to HDs and contains an extracellular collagen-like sequence [20]. The cytoplasmic plaque of HDs also contains intermediate filaments [11], a newly identified 200-kDa component [21], and HD-1, a high molecular mass protein [22].

Basal cell adhesion to a substratum is mediated by FAs in addition to HDs [3,23]. Keratinocytes in culture utilize $\beta1$ integrins in FAs as the primary adhesion receptor involved in recognition of extracellular ligands [3]. In contrast to HDs, FAs are readily identified in cell culture but have not been identified in skin. The differences in detectability of HDs and FAs in tissue and culture may be due to differences in stability of the adhesion structures as well as function. FAs are relatively labile structures that are short-lived and may not be easily detected in normal epidermis. In contrast, HDs are stable structures [14]. Consistently, when anchored cells are induced to migrate, as in wound repair, $\alpha6\beta4$ and BPAG1 relocate from HDs to a diffuse distribution over the cell surface and cytoplasm [24]. The cytoplasmic tail of the integrin $\beta4$ subunit in HDs is larger (1000 amino acids) than that of the $\beta1$ integrin (50 amino acids) [19]. The unique cytoplasmic sequences of the $\beta4$ and $\beta1$ subunits regulate associations of the HDs and FAs with keratin and actin filaments, respectively, through their associated cytoplasmic linker proteins.

Despite the instability of FAs relative to HDs, FAs are formed in cultured cells only after initial adhesion, cell spreading, and migration have ceased. Adhesion structures that mediate initial adhesion, spreading, and migration are not detectable by light microscopy and are not well-characterized. However, they are probably structurally and functionally similar to FAs. This suggested similarity is based on the fact that anti-integrin antibodies that block or activate initial cell adhesion, spreading, and migration react with the same integrin receptors that localize in FAs [25]. Although the labile adhesion structures that mediate migration contain integrins and actin filaments, a cascade of regulatory factors and cytoskeletal linker proteins may modulate the function of the integrins and actin filaments and may distinguish migration adhesion structures from FAs. The role of integrins and actin-containing filaments and associated cytoskeletal components in regulating locomotion has recently been reviewed [26]. Vesicular traffic may provide adhesion receptors in tips of lamella of migrating cells that are subsequently incorporated into adhesion structures and are eventually endocytosed behind the leading edge of the cell.

In FAs, the $\beta1$ or $\beta3$ subunit of the integrin heterodimer receptors are associated directly with cytoplasmic talin or α-actinin and indirectly with vinculin, paxillin, zyxin, tensin, and a series of regulatory components including the tyrosine kinases, pp60src, and $p125^{FAK}$ [27]. For example, overexpression of vinculin suppresses cell motility [28]. Phosphorylation of tyrosine residues in tensin, zyxin, paxillin, and $p125^{FAK}$ has been implicated as a regulatory signal for assembly/disassembly of FAs [27].

Cadherin adhesion receptors are involved in cell-cell adhesion of basal cells but are not directly involved in basal cell adhesion to the BM and will only be discussed briefly. Cadherins are Ca^{++}-dependent mediators of homotypic cell-cell adhesion and are the primary cell-cell adhesion receptors in the epidermis [29]. There are two major classes of cadherins termed classical cadherins and desmosomal cadherins that are components of adherens junctions and desmosomes, respectively [9,29]. The two types of cadherins exhibit a high degree of sequence homology in their extracellular domains but differ in their cytoplasmic sequences that associate with the cytoskeleton. Epidermal cells express cell-cell adherens junctions that contain classic cadherins that associate with actin filaments. P cadherin is expressed primarily in the basal cell layer whereas E cadherin is expressed in basal, suprabasal, and spinous cells [30].

Epidermal cells also form extensive cell-cell adhesions of the desmosomal type [9] that facilitate the epidermal function as a permeability barrier. Desmoglein [31] and desmocollins [32] are adhesion components of the desmosomes and are referred to as desmosomal cadherins [9,29]. Desmosomes are relatively few in the basal cell layer but are upregulated in the suprabasal and spinous cell layer. Consistently, serum from patients with the autoimmune blistering

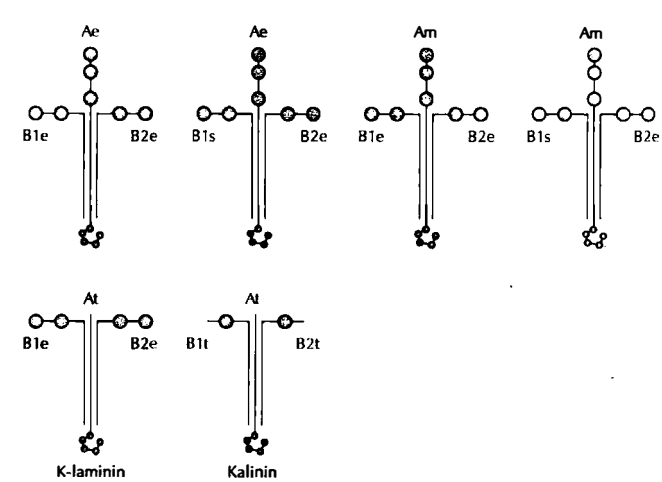

Figure 1. Schematic illustration of laminin variants. The isoforms Ae-B1e-B2e, Ae-B1s-B2e, Am-B1e-B2e, and Am-B1s-B2e have been isolated from several tissues. The exact nature of the kalinin (epiligrin/nicein) and K-laminin isoforms remains to be established, as the tentatively genetically distinct truncated At and B1t chains have not yet been completely sequenced. Reprinted from [40].

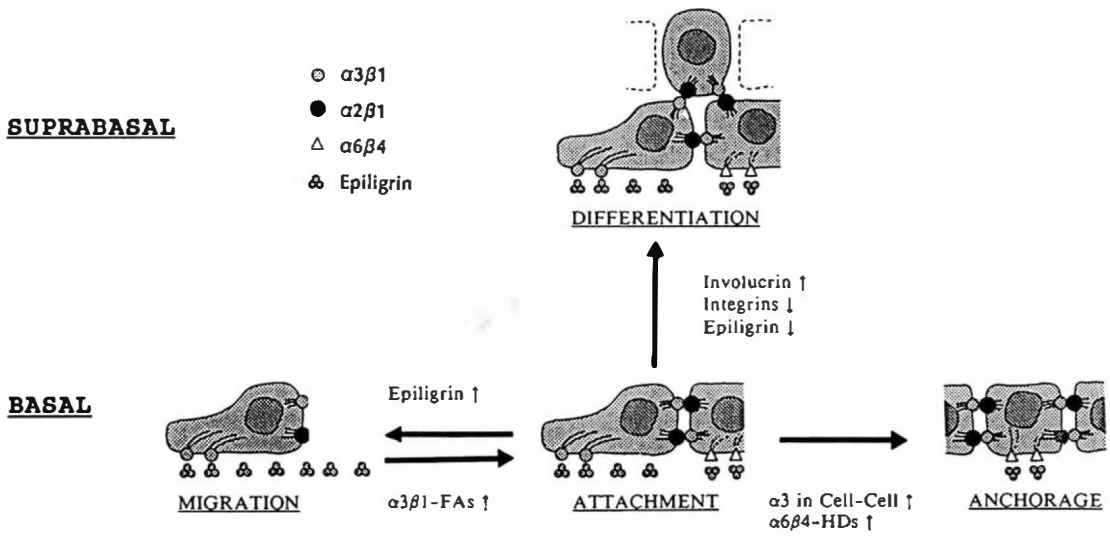

Figure 2. Overview: sequential roles for integrins α3β1 and α6β4 in adhesion of epidermal basal cells to epiligrin in the BM. Our current understanding of the sequential expression and function of integrins α3β1 and α6β4 adhesion structures is summarized diagrammatically for basal and suprabasal cells layers of the epidermis. Basal layer: basal keratinocytes synthesize, attach, and spread on epiligrin in the BM via α3β1 that associates with actin containing stress fibers. In culture, progressive changes in adhesion structures correlate with decreasing migration, increased cell-cell contact, and increased stability of the cell-substrate adhesions. Initial attachment, spreading, and migration (see Migration) precede formation of α3β1 in FAs (Attachment). The formation of α3β1-FAs functions as a nucleation site for the reorganization of α6β4 into stable HDs (Anchorage). Concurrent with the formation of the HDs, α3β1 is upregulated in cell-cell contacts and functions in cell-cell adhesion with α2β1 as a co-receptor as previously described [44]. We suggest that regulation of adhesion to epiligrin in the BM via α3β1 and α6β4 plays a major role in regulation of differentiation and activation in wound repair. Epiligrin expression is upregulated (↑) in migrating and proliferating cells at the edge of wounds or colonies in culture or tissue [46]. Conversely, increased cell density results in decreased synthesis of epiligrin (↓), increased formation of stable α3β1-FAs followed by increased α6β4-HDs, and α3β1 and α2β1 at cell-cell contacts. Suprabasal layer: In culture, detachment (Detachment) of basal cells from the BM with inhibitory antibodies to α3β1 induces α3β1-mediated cell-cell adhesion [44] and expression of involucrin,‡ a component of the granular cell layer (Differentiation). This suggests that cell adhesion to epiligrin inhibits epidermal differentiation whereas detachment induces α3β1 function in cell-cell adhesion as a normal differentiation step. Detachment of basal cells results in downregulation of integrins and epiligrin.

disorder, pemphigus vulgaris (PV), express autoantibodies that cause detachment of suprabasal cells from the basal cells. Recently the PV antigen was shown to be identical to desmoglein, a desmosomal cadherin [33]. The PV antigen, like the desmosome, is upregulated in the suprabasal cell layer where it is a target for the autoimmune antibodies.

The Basement Membrane Gipson *et al* [34] suggested that formation of HDs by basal cells may be controlled by the components of the BM. The unique organization of the epidermal BM in proximity to HDs supports its possible regulatory role in HD formation [35]. The synthesis and assembly of the BM is a cooperative effort of epidermal and dermal cells. In the epidermal BM, type VII collagen-containing anchoring fibrils in the dermis extend into the lamina densa of the BM adjacent to HDs [36]. Anchoring filaments extend from the anchoring fibrils across the lamina lucida, to the HDs. These BM structures may function as nucleation points for formation of FAs and HDs via integrin adhesion receptors. The composition of the anchoring filaments is being established and may include a number of related glycoproteins; epiligrin [3], kalinin [7], a 125-kDa BM component [37], and the high-molecular-mass antigen complex recognized by the GB3 monoclonal antibody (MoAb), termed nicein [5]. Recently epiligrin [4] and kalinin [38] were shown to be related to nicein. It is likely that epiligrin/kalinin/nicein are related to a unique truncated laminin chain, termed B2t, that was recently cloned and sequenced [39].

Multiple variant forms of laminin are present in the lamina lucida of the epidermal BM [39-42] and include epiligrin, K-laminin described by the Burgeson lab [42], and EE laminin that may be related to K-laminin (see later discussion). A recent review by Tryggvason [40] has described the known and putative subunit compositions of the laminin variants and is summarized in **Fig 1**. K-laminin is a laminin isoform that is co-purified with kalinin and may be immunologically related to kalinin. K-laminin contains laminin B1e and B2e subunits and a third alternate A chain or At subunit [40] that is immunologically related to the BM165 chain of kalinin [42]. Additional components of the BM include entactin/nidogen, heparan sulfate proteoglycan, and type IV collagen [35]. Epiligrin ([3] and see later discussions), laminin variants, collagen, and entactin/nidogen have all been shown to interact with integrins *in vitro* and may contribute to basal cell adhesion to the BM *in vivo* (for review see [17]). This suggests that basal cell adhesion to the BM may involve one or more different receptors, ligands and anchoring junctions.

EPILIGRIN, AN EPIDERMAL BASEMENT MEMBRANE LIGAND FOR INTEGRINS α3β1 AND α6β4

Differential and Polarized Expression of Integrins In normal skin, expression of the major integrins, α6β4, α3β1, and α2β1, is restricted to the proliferating basal/suprabasal cell layers. An outline for the possible unique functions for the different integrins is presented diagrammatically in **Fig 2**. The distribution of the integrins in normal epidermis and epidermis from donors with JEB is presented in **Fig 3**. All three integrins are down-regulated in the differentiated spinous cells [15,16,43]. For example, during suspension-induced terminal differentiation of keratinocytes, the α5β1 integrin is also down-regulated and this occurs in two stages. First, the ability of the receptor to bind fibronectin declines. Second, mRNA levels reduce and the quantity of receptor declines. The decline reflects both inhibition of subunit transcription and inhibition of maturation and intracellular transport of subunits [15]. Expression of α6β4 is restricted to the basal plasma membrane ([3,17] for review) except in developing epidermis prior to formation of HDs. In contrast, α3β1 is expressed on the basal, lateral, and apical

‡ Symington BE, Carter WG: Modulation of epidermal differentiation by epiligrin and integrin α3β1 (manuscript submitted).

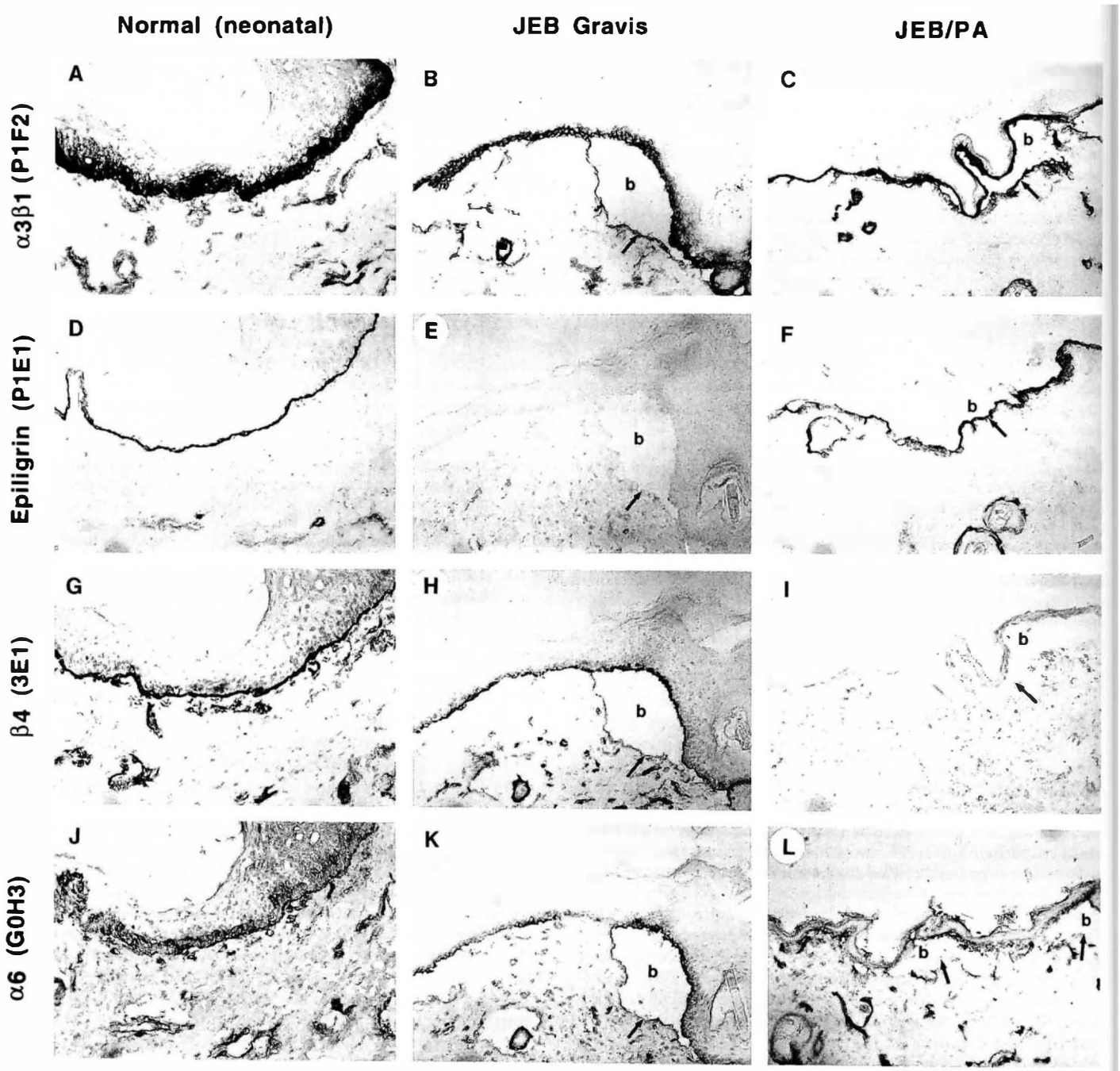

Figure 3. Immunoperoxidase Staining of Cryostat Sections of Skin From a Healthy Neonate, a JEB Gravis Patient and a JEB Patient With Pyloric Atresia (JEB/PA). Skin sections from a healthy neonate (panels *A,D,G,J*) a JEB gravis patient *(B,E,H,K)* and a JEB/PA patient *(C,F,I,L)* with indicated MoAb for $\alpha3\beta1$ *(A–C)*, epiligrin *(D–F)*, $\beta4$ subunit *(G–I)*, and $\alpha6$ subunit *(J–L)*. Sections from the two individuals with JEB display both areas of epidermal/dermal attachment and detachment with small blisters *(b)*. The patient with JEB and JEB/PA exhibited no detectable HDs by electron microscopy. The basement membrane region associated with the dermal side is demarcated by *arrows*. The individual with JEB gravis alone exhibits an absence of epiligrin *(E)*, which has previously been associated with the JEB gravis (or Herlitz) subgroup. In contrast, the fetus diagnosed with JEB/PA has normal epiligrin expression *(F)*, but an absence of detectable $\beta4$ subunit *(I)* and reduced $\alpha6$ subunit *(L)* expression at the basal surface of the basal epidermal cells.

membranes whereas $\alpha2\beta1$ is expressed primarily on the apical and lateral membranes [44]. This suggests that $\alpha3\beta1$ functions in both cell-cell and cell-BM adhesion in normal epidermis. $\alpha3\beta1$ has been shown to mediate cell-cell adhesion as well as cell substrate adhesion to epiligrin [3,23,44].

Epiligrin, A Ligand for Cell Adhesion to the BM Epiligrin is an effective ligand for adhesion of keratinocytes and other cells to the substratum via integrin $\alpha3\beta1$ [3,44,45]. In addition, epiligrin co-distributes with integrin $\alpha6\beta4$ in HD-like SACs [3], suggesting that it may function as a ligand for $\alpha6\beta4$. In culture, $\alpha6\beta4$ forms a stable complex with exogenous HFK-ECM [3,14] indicating that exogenous epiligrin controls the organization of endogenous $\alpha6\beta4$. Further, JEB and epiligrin cicatricial pemphigoid, an acquired autoimmune blistering disorder, appear to be due to disorders of epiligrin ([4] and see later discussion). Together these results indicate that epiligrin interacts directly with $\alpha3\beta1$ and directly or indirectly with $\alpha6\beta4$ in HDs, and these interactions are instrumental in basal cell attachment to the BM. Recently, we have shown that disruption of basal cell interactions with epiligrin with anti-$\alpha3\beta1$ MoAb

Table I. Characteristics of New Anti-Epiligrin Monoclonal Antibodies

	Reaction of the Antibody with										
		Tissue[b]									
		Normal		JEB		Epiligrin[c]		EE-LAM[d]	Attachment Inhibition[e]		
Antibody	HFK-ECM[a]	Epi	End	Epi	End	Blot	Rip	Rip	Epi	Col	Fn
Epiligrin/EE-Laminin Specific											
P1E1	+	+	±	−	±	−	+	+	−	−	−
G3-3-SG	+	+	+	+	+	−	+	+	−	−	−
D3-4-SG	+	+	+	+	+	−	+	+	±	−	−
P3H9-2-EW	+	+	+	+	+	−	+	+	+	−	−
Epiligrin-Specific											
P3C10-A6	+	+	−	−	−	+	+	−	−	−	−
C2-9-SG	+	+	−	−	−	−	+	−	++	−	−
B4-6-SG	+	+	−	−	−	+	+	−	−	−	−
P3E4-EW	+	+	−	−	−	+	−	−	−	−	−
GB3	+	+	−	−	−	−	+	−	−	−	−

[a] Immunofluorescence microscopy of ECM from cultured HFKs.
[b] Immunoperoxidase staining of normal and JEB gravis skin (sample: SA 8-14-92); epidermal (Epi) and endothelial (End) BM.
[c] Immunoblot (Blot) or immunoprecipitation (Rip) reaction with epiligrin after affinity purification from HFK culture supernatant.
[d] None of the antibodies react with human placental laminin. However, epiligrin/EE-laminin antibodies co-precipitate a laminin-like protein of 190/230, referred to as EE-Laminin (EE-LAM).
[e] MoAb C2-9-SG >P3H9-2-EW > D3-4-SG inhibit cell adhesion to epiligrin (Epi) but not collagen (Col) or Fibronectin (Fn).

induces expression of involucrin,‡ a late-stage differentiation component involved in formation of the cell envelope **(Fig 2)**. This suggests that interactions of the basal cells with epiligrin via $\alpha3\beta1$ and possibly $\alpha6\beta4$ may play a role in regulating proliferation, differentiation, and stratification in the epidermis.

Epiligrin contains three disulfide-bonded, non-collagenous, glycoprotein subunits, E170, E145, and E135, based on molecular mass in kDas [3]. Two additional glycoproteins, E200 and E100, are variably associated with epiligrin and are a precursor to E170 and a degradation product of the E145 subunit, respectively. We prepared MoAb P1E1 that reacts with E170, and was used for the initial immunopurification of epiligrin from HFK culture medium and for localization studies. Immunolocalization in tissue identified epiligrin in the dermal-epidermal junction of epithelial cells in organs primarily of endodermal/ectodermal origin. At the ultrastructural level, epiligrin localized to the lamina lucida of BMs.

We compared cell adhesion to epiligrin via integrin $\alpha3\beta1$ in focal adhesions (FAs) with $\alpha6\beta4$ in hemidesmosomes (HDs) as mediator of distinct epithelial cell adhesion and signaling functions.§ $\alpha3$-transfected K562 cells, fibroblasts, HT1080 fibrosarcoma cells, and melanocytes attach and spread on epiligrin via $\alpha3\beta1$. Both attachment and motility on epiligrin are blocked with monoclonal antibodies (MoAbs) against $\alpha3$ (P1B5), $\beta1$ (P4C10), epiligrin (C2-9), or at 4°C, whereas attachment and motility of human foreskin keratinocytes (HFKs) on epiligrin involves both $\alpha3\beta1$ and $\alpha6\beta4$. Anti-$\alpha3$ MoAb blocks cell spreading but not attachment. In contrast, anti-$\alpha6$ (G0H3) was unable to block either attachment or spreading but inhibited HD formation as evidenced by inhibition of localization of bullous pemphigoid antigen I (BPAI) in HDs. When anti-$\alpha3$ and anti-$\alpha6$ were combined they blocked both attachment and spreading of HFKs on epiligrin [14]. We then evaluated HFK adhesion to epiligrin via $\alpha6\beta4$ at 4°C, a condition that inhibits $\alpha3\beta1$-dependent adhesion. Attachment of HFKs at 4°C occurs without spreading and is blocked with anti-$\alpha6$ MoAb indicating that $\alpha6\beta4$ mediates attachment independent of $\alpha3\beta1$. Both attachment of HFKs via $\alpha6\beta4$ and spreading via $\alpha2\beta1$ are blocked with anti-epiligrin MoAb (C2-9) confirming the dual roles of epiligrin in adhesion via both receptors. Anchorage of HFKs to epiligrin via $\alpha6\beta4$ is distinct from adhesion and spreading on epiligrin, fibronectin, or collagen via $\beta1$ integrins because i) it is more rapid than adhesion via $\beta1$ integrins and occurs at 4°C; ii) it does not result in cell spreading. Thus, epithelial cells, in contrast to many other cells that express only $\alpha3\beta1$, control motility and stable anchorage on epiligrin by differential use of $\alpha3\beta1$ and $\alpha6\beta4$ in HDs, respectively, resulting in distinct transmembrane signals.

Identification of EE-laminin and Its Relation to Epiligrin

Immunoprecipitation of the epiligrin heterotrimer E170 (putative laminin At subunit), E145/E100 (laminin B2t), and E135 (putative laminin B1t) from conditioned culture medium of HFKs with MoAb P1E1 also precipitated proteins of 230 and 190 kDa [3]. The 230/190 protein was described as laminin-like because it could be removed by precipitation with anti-laminin antibodies that did not precipitate epiligrin. Thus, epiligrin was defined as the heterotrimer (E170, E145/E100, E135) that was immunoprecipitated with P1E1 after preclearing with anti-laminin antibodies [3]. We suggested that the laminin-like complex is either bound to epiligrin and/or cross reacts with the P1E1 MoAb. For simplicity in the following discussion we will refer to the 230/190 laminin-like complex as EE-laminin because it is expressed in BM of both epidermis (E) and microvascular endothelium (E). Conceivably, EE-laminin may be related to K-laminin described by Marinkovich *et al* [42]. However, neither antibody nor cDNA probes specific for K-laminin or EE-laminin are currently available to evaluate their relationship at this time.

We have identified seven new MoAb that react with epiligrin [45]. These MoAb have been categorized as specific for epiligrin or epiligrin/EE-laminin based on differences in results from immunoprecipitation and tissue staining. The characteristics of these MoAb are summarized in **Table I** and **Fig 4** and as follows: i) all seven MoAb react with epiligrin as determined by preclearing with anti-epiligrin P1E1 MoAb; ii) three of the MoAb inhibit melanocyte, keratinocyte, T-cell, and fibroblast adhesion to epiligrin but not collagen, laminin, or fibronectin; iii) epiligrin/EE-laminin–specific MoAb, including the original P1E1 MoAb, precipitate both the 190/230 kDa EE-laminin and the three subunits of epiligrin (E170, E145/E100, E135); iv) epiligrin-specific MoAb precipitate epiligrin but not EE-laminin; v) in skin, epiligrin-specific MoAb stain only epidermal BM whereas epiligrin/EE-laminin–specific MoAb stain epidermal and endothelial BM in the microvasculature **(Fig 4)**.

These results indicate that EE-laminin is present in BMs of the epidermis and microvascular endothelium whereas epiligrin is expressed only in epidermal BMs. These results also indicate that epiligrin and EE-laminin share multiple common epitopes recog-

§ Gil SG, Xia Y, Takada Y, Carter WG: Functions for integrins $\alpha6\beta4$ in anchorage and $\alpha3\beta1$ in motility of keratinocytes on epiligrin (manuscript submitted).

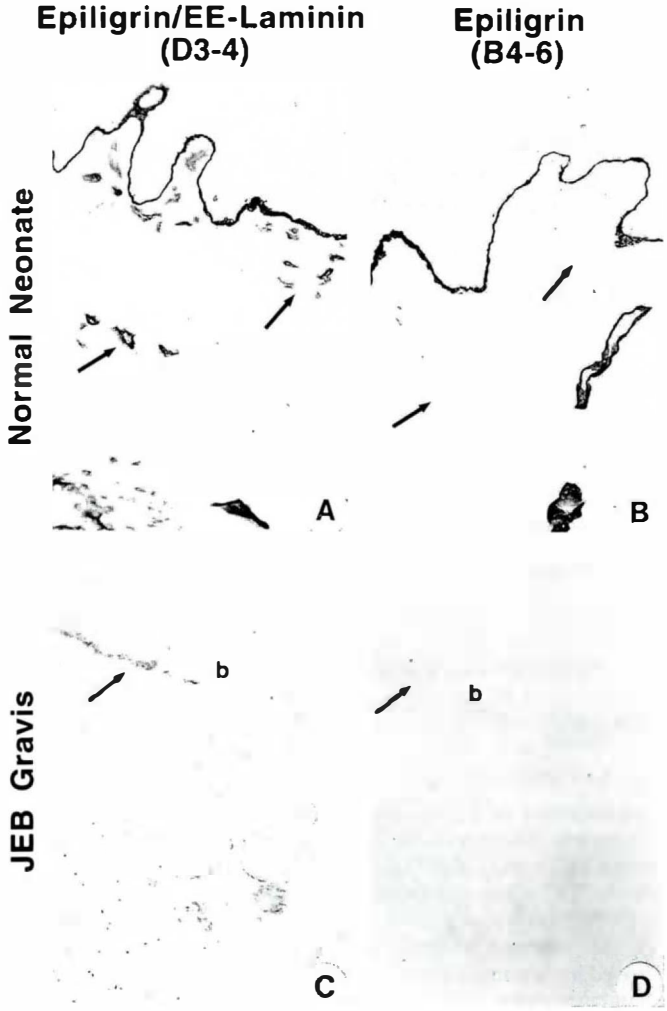

Figure 4. Immunostaining of Cryostat Sections of Skin From a Normal Donor, and a Patient with JEB Gravis With MoAb Specific For Epiligrin and Epiligrin/EE-Laminin. Immunoperoxidase staining of cryostat sections of skin from a healthy neonate *(A,B)* and a JEB gravis patient *(C,D;* individual SA 8-14-92 in **Table II**) with either MoAb D3-4 recognizing both epiligrin and EE-laminin *(A,C)* or the epiligrin-specific MoAb B4-6 *(B,D)*. Sections from the individual with JEB gravis display both areas of epidermal/dermal attachment and detachment with small blisters *(b)*. *Arrows* indicate either the location of the microvasculature in the dermis of normal neonatal skin *(A,B)* or the location of the epidermal basement membrane in JEB gravis skin *(C,D)*. In the healthy control, the B4-6 staining is restricted to the epidermal basement membrane *(B)*, whereas D3-4 also detects the basement membrane in the microvasculature *(A)*. In the JEB gravis patient, some staining with D3-4 *(C)*, but not B4-6 *(D)*, is noticeable in the basement membrane region.

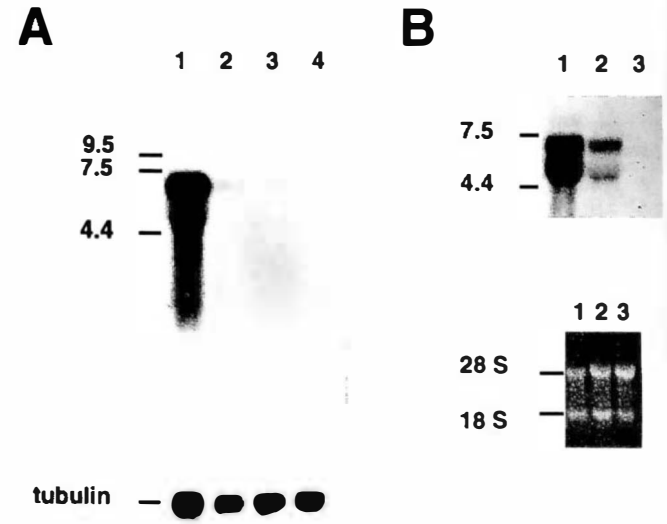

Figure 5. Northern Blot Analysis of Total Cellular RNA Isolated From Human Foreskin Keratinocytes and Fibroblasts and Northern Blot Analysis of RNA Isolated From Proliferating and Differentiating Keratinocytes. *A)* Keratinocytes *(lane 1)*, FEP18-11 *(lane 2)*, FEP1L-8 *(lane 3)*, and human foreskin fibroblasts *(lane 4)*. FEP18-11 and FEP1L-8 are human foreskin keratinocytes that have been transformed with human papilloma virus. Hybridization with an epiligrin-specific cDNA probe detects two transcripts, approximately 5 and 6 kb in size, which are significantly decreased in the cell lines that have been transformed with papilloma virus *(lanes 2,3)*. No signal is detectable in human foreskin fibroblast RNA *(lane 4)* that was included as a negative control. Positive hybridization to tubulin mRNA shows that equal amounts of RNA were loaded in *lanes 1–4*. *B*, Proliferating keratinocytes *(lane 1)*, keratinocytes treated with 1.3 mM of Ca^{++}, *(lane 2)*, and confluent keratinocytes *(lane 3)*. The reduced signal in *lanes 2,3* show that the epiligrin transcript is significantly decreased by factors that cause keratinocyte differentiation. Photograph of 28S and 18S RNA shows that equal amounts of RNA were loaded in *lanes 1–3*.

nized by a number of MoAb. Thus, the E170 subunit of epiligrin and at least one subunit of EE-laminin are immunologically related. EE-laminin and epiligrin can be immunoprecipitated independently, indicating that they are not covalently associated in the conditioned tissue culture media (Gil and Carter, manuscript in preparation).

We have identified and sequenced multiple cDNA clones encoding the 170-kDa subunit of epiligrin [46]. Consistent with the immunologic data above, partial sequence analysis of the cDNA clones for E170 revealed a homology with domains IIIa and II of human laminin A chain. Domain IIIa encodes a cysteine-rich region containing multiple EGF-like repeats [41]. Sequence alignment of E170 and human laminin A chain in domain IIIa showed conservation of the cysteine residues and 55% sequence homology. In contrast to domain III, the primary amino acid sequence in domain II is less well-conserved between E170 and laminin A chain. However, a structural relationship is maintained in that both proteins encode alpha-helical domains characteristic of a coiled-coil structure previously described for laminin [41]. Complete sequence analysis will be necessary to determine how E170 relates to the other domains present in laminin.

Regulation of Epiligrin Expression: Effects of Cell Density, Wounding, and Oncogenic Transformation Northern blot analysis of HFK mRNA with a cDNA probe (**Fig 5*A***; Ep-1 transcript) specific for epiligrin identified at least two mRNAs of 5 and 6 kb. These sizes are compatible with the expected mRNAs coding for the E170 subunit of epiligrin. It is possible that the two mRNA transcripts correspond to the E170 subunit of epiligrin and the immunologically-related subunit of EE-laminin. Northern blot analysis of HFK mRNA and mRNA from HPV transformed HFKs (FEPE1L-8 cells) identified reduced amounts of epiligrin mRNA in the FEPE1L-8 cells. This confirms and extends our published report that transformation by HPV causes decreased expression of HFK ECM [47].

In human wounds, we have observed that epiligrin is deposited in BM localized to the leading edge of the migratory tongue of epithelium 24, 48, and 72 h after wounding in normal human volunteers [46]. In these wounds, epiligrin and $\alpha 3\beta 1$ were polarized to the basal surface of cells in the migrating epithelial tongue. *In situ* hybridization with anti-sense cRNA probes specific for epiligrin identified elevated epiligrin mRNA in the wound site. These data suggest that $\alpha 3\beta 1$ and epiligrin may be of primary utility during epidermal migration and wound repair, possibly in relation to the

Table II. Screening Results for Skin Samples from Patients with Epidermolysis Bullosa[a]

Clinical Classification (Patient)	Biopsy Source	Antigen		
		α3β1 (P1F2)	α6β4 (3E1)	Epiligrin (P1E1)
Normal				
Fetal epidermis		+	+	+
Neonatal Foreskin		+	+	+
Adult		+	+	+
Junctional				
Mitis				
EB86-2A (sib)	Sybert/Smith	+	o	+
EB86-2 (sib)	Sybert/Smith	+	o	+
Gravis				
PNDX91-4	Folk/Holbrook	+	+	−
YS 8-14-92	Yordy/Sybert	+	+	−
With Pyloric Atresia				
EB92-11	Dolan/Sybert	+	− ($\alpha6^+\beta4^-$)	+
Simplex				
S 7-1-91 (+muscular dystrophy)	Sybert	+	+	+
EB91-9	Sybert/Smith	+	+	+
Dystrophic				
EB91-4	Sybert/Smith	+	+	+

[a] −, not detectable by immune peroxidase staining of crystat sections; o, expression with altered organization.

deposition and assembly of epiligrin in the BM. In contrast, laminin and α6β4 were reduced in cells at the leading edge of the migratory epidermal tongue, consistent with the role of HDs as stable adhesion structures for non-migrating cells [24]. In related results, northern blot analysis of mRNA from HFKs grown at low density versus confluence identified a dramatic increase in expression of epiligrin mRNA in sparse cells **(Fig 5*B*)**. This indicates that epiligrin mRNA is upregulated at low cell densities as occurs at the wound edge. Together, these results indicate that expression of epiligrin is upregulated by wounding or reduced cell-cell contact and downregulated by detachment from the substratum. It should be noted that the decreased expression of epiligrin in transformed cells contrasts markedly with the observed increase in epiligrin in wounds. Thus, the migration of wound cells may be self-limiting by the production of a restricting BM. In contrast, the migration of transformed cells in a wound environment would have no such limitation.

Overall, epiligrin expression at the mRNA levels is relatively low in normal homeostatic epidermis. Wounding, tissue culture, TGFα, and TGFβ each upregulate epiligrin expression. In contrast, detachment induced differentiation or immortalization with E6 and E7 genes from HPV downregulate expression of epiligrin [46].

REDUCED EXPRESSION OF EPILIGRIN AND INTEGRIN β4 IN JUNCTIONAL EPIDERMOLYSIS BULLOSIS

We have identified both acquired [4] and inherited epidermal blistering disorders that may be caused by defects in the adhesive function and/or expression of epiligrin and the β4 integrin subunit. Results from these studies support the proposed functions for epiligrin and β4 as physiologically significant components of HDs and as mediators of basal cell adhesion to the BM *in vivo*.

Epiligrin is related to nicein [4], a BM protein that is decreased or absent in BM of patients with the gravis form of JEB. Consistently, we have observed decreased expression of epiligrin as detected with MoAb P1E1 in patients with JEB gravis **(Fig 3)**, but not milder localized forms of JEB, simplex, or dystrophic EB, as previously reported [3]. The results from these studies are summarized in **Table II** and are consistent with published results for nicein [6]. Cultured keratinocytes from one of the JEB gravis patients were weakly attached and did not spread when compared to cultured keratinocytes from normal donors. Consistent with the absence of epiligrin in the BM, the patients' cells failed to deposit epiligrin into the ECM as detected by immunofluorescence microscopy with anti-epiligrin MoAb (Brown *et al,* manuscript submitted).

Immunostaining of the JEB skin samples with MoAb specific for epiligrin or epiligrin/EE-laminin confirmed the results in both **Table I and Table II**. Epiligrin was found to be completely absent from both the BM of patients with JEB gravis and BM of microvasculature when detected with MoAb specific for epiligrin **(Fig 4)**. In contrast, MoAb specific for epiligrin/EE-laminin were reduced in the epidermal BM but still clearly detectable, suggesting the continued expression of EE-laminin in the epidermal BM **(Fig 4)**. Recently, two JEB patients with mutations in the LAMB2T gene were identified (J. Uitto and K. Tryggvason, personal communication). One of their patients had a homozygous splice site mutation in the LAMB2T gene that resulted in deletion of exon 9. A second patient had a heterozygous deletion-insertion mutation in the LAMB2T gene that resulted in premature termination. This data suggests that mutations in the LAMB2T gene may be causal in some forms of JEB.

We also examined skin samples from a fetus diagnosed with JEB gravis, combined with pyloric atresia (PA), or incomplete formation of the pyloris. Ultrastructural analysis of the epidermis from this patient detected severely reduced numbers of HDs at the epidermal junction with the BM [48], consistent with the clinical diagnosis of JEB gravis. Surprisingly, skin samples from this patient expressed normal levels of epiligrin (**Fig 3**, JEB/PA). Consistent with this result, four different cases of combined JEB with PA have been reported to be positive for nicein/epiligrin [49–52]. This clinical phenotype may define a pleiotropic effect of a single gene or defects in two closely linked genes. Further analysis of this skin sample identified normal α3 and α6 expression but absence of detectable β4 (**Fig 3**, JEB/PA; Brown *et al,* manuscript in preparation). This phenotype of $\beta4^-$, epiligrin$^+$, may define a new variant of JEB that is distinct from JEB gravis which is epiligrin$^-$, $\beta4^+$. Further, these results testify to the physiologic significance of β4 in formation of HDs. Defects in either epiligrin or β4 result in reduced HD formation and epidermal blistering at the lamina lucida.

In situ hybridization of skin sections from the JEB/PA patient with β4 cRNA probes suggests that mRNA specific for β4 continues to be expressed in basal epidermal cells although β4 protein is not detectable (Brown *et al,* work in progress). This suggests that the β4 mRNA encodes a mutation that results in decreased synthesis, in-

creased turnover, or failure of $\beta 4$ protein to incorporate into the HDs.

JEB is a genetically heterogeneous blistering disorder with possible inherited mutations in epiligrin and integrin $\beta 4$. These defects result in inadequate expression of epiligrin and $\beta 4$, incomplete assembly of HDs resulting in blister formation. These results testify to the physiologic significance of epiligrin and $\beta 4$ in HD formation. Additional information about i) the molecular characteristic of the inherited mutations, ii) the effects of these adhesion defects on the development biology of the affected tissues, and iii) the role of other components, such as K-laminin, EE-laminin, and BPAG2, in the function of HDs are interesting areas for future studies. These studies may provide clinical approaches for treatment of the pathologic effects of this form of blistering disease.

This work was supported by grants from the National Institutes of Health, RO1-CA49259 (WGC), PO1-AR21557 (WGC), and F32-GM15085 (MCR), and the American Cancer Society, CB-591 (WGC).

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